AD_____
(Leave blank)

Award Number: W81XWH-09-2-0070

TITLE: Corneal protection for burn patients

PRINCIPAL INVESTIGATOR: Irene E. Kochevar, Ph.D.

CONTRACTING ORGANIZATION:

Massachusetts General Hospital
Boston MA 02114-2621

REPORT DATE: October 2013

TYPE OF REPORT: Final report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

☒ Approved for public release; distribution unlimited

☐ Distribution limited to U.S. Government agencies only; report contains proprietary information

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE *(DD-MM-YYYY)* | 2. REPORT TYPE | 3. DATES COVERED *(From - To)* |
|---|---|---|
| October-2013 | Final report | 1 July 2009 – 31 July 2013 |

**4. TITLE AND SUBTITLE**
Corneal protection for burn patients

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-09-2-0070

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**
Irene E. Kochevar

**5d. PROJECT H -09-2-0070NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

Massachusetts General
Hospital, Boston
Massachusetts 02114-2621

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
US Army Medical Research and
Material Command
Fort Detrick Maryland
21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for public release; distribution unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

**Purpose:** The overall goal of this research is to preserve vision of patients recovering from severe facial burns by providing an improved method to reduce development of corneal defects, inflammation, infection and opacification. **Scope:** To improve and understand the properties of the degradation-resistant crosslinked amniotic membranes for treating cornea of burn patients, to evaluate a sutureless method for attaching membranes to cornea and construct a membrane-encapsulated contact lens. **Major findings**: Identified three crosslinking methods that produced amnion with maximum protection against enzymatic degradation while remaining flexible enough to conform to cornea shape. Determined that protein crosslinking greatly reduces availability of beneficial factors in native amnion. Selected two crosslinking methods for in vivo evaluation. Demonstrated that crosslinked, degradation-resistant amniotic membrane can be securely sealed to the corneal surface using a sutureless, light-activated technique. Constructed an amnion-encapsulated hydrogel lens to maintain hydration on the cornea surface.

**15. SUBJECT TERMS**
ectropion, cornea, burn scars, amniotic membrane, protein crosslinks, photomedicine, photochemistry, proteinases

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRMC |
| U | U | U | UU | 16 | 19b. TELEPHONE NUMBER *(include area code)* |

## Table of Contents

## INTRODUCTION

The overall goal of this research was to improve the visual outcomes as well as the quality of life for burn patients during the acute and convalescent phases of their rehabilitation. Scarring from second and third degree facial burns, and from subsequent skin grafts, causes the tissues involved to contract and, if significant enough, the patient is left unable to blink or close their eyes. This results in desiccation of the ocular surface, breakdown of the cornea's defense mechanisms and subsequent events that may lead to cornea opacification and the need for cornea transplant. Currently these patients receive frequent application of artificial tears, an imperfect solution that slows down their rehabilitation. Amniotic membrane (AM) transplantation to the ocular surface can assist in the maintenance of the ocular surface of these patients. However, commercially available membrane is not only very expensive, but enzymes on the inflamed ocular surface "dissolve" the AM very rapidly; in one day compared to two weeks in non-burn patients. In this project, our major goal is to stabilize the AM to allow it to be used to protect the corneas of burn patients. Our approach was to crosslink the constitutive proteins in AM before applying it to the patient's eye. Studies in tissues have shown that crosslinking proteins protects them from enzymatic degradation. The majority of these studies focused on identifying the crosslinking method that most effectively decreases the rate of enzymatic degradation of AM while preserving the beneficial factors in AM. In addition, we evaluated photobonding as a sutureless, glueless alternative to sutures for attaching crosslinked AM to cornea. We also carried out pilot studies to test an approach that combines AM with a hydrogel material to increase the ability of the amnion to hydrate the cornea. The in vivo studies employ a rabbit model of eye inflammation.

## BODY

This Grant Agreement was for a joint project with Col Anthony J. Johnson, MD, PI on Grant Agreement W81XWH-09-2-0069. The Statement of Work included tasks to be carried at both the Massachusetts General Hospital (in vitro studies) and the Brooke Army Medical Center (later at USA Institute for Surgical Research; in vivo studies). Dr. Kochevar and Dr. Johnson communicated frequently by phone, reciprocal visits and discussions at conferences during the course of these studies. Dr. Johnson has received an extension of the grant period and will submit a final report separately.

Brief summary

Six methods were evaluated for their ability to crosslink proteins in amniotic membrane (AM) and produce a material suitable for application to the corneas of burn patients to prevent desiccation. The criteria used to select material to be tested in vivo were: 1) ability to inhibit enzymatic degradation of AM, 2) good pliability, 3) preservation of beneficial small proteins in AM and non-toxicity. The crosslinking methods selected were treatment with a carbodiimide-containing reagent (EDC) and Rose Bengal photosensitization. Excellent or moderate resistance to enzymatic degradation was achieved and a pliable crosslinked material was produced. However, none of the methods tested fully preserved the beneficial factors in AM during crosslinking. Multilayer crosslinked AM constructs inhibited enzymatic degradation but were too stiff to apply over the cornea. Crosslinked AM could be photochemically bonded to the cornea as an alternative to suture attachment. An hydrogel contact lens was encapsulated between AM layers for maintaining cornea hydration was constructed in a pilot study.

### Specific Aim 1. To modify amnion to make it less susceptible to degradation while preserving its anti-inflammatory and healing properties.

1.a. Determine the relationship between extent of protein crosslinking and the degradation rate of amnion in vitro. We evaluated physical and chemical protein cross-linking methods that

operate by different molecular mechanisms and produce different covalent crosslinks. Human amniotic membrane (AM), harvested from scheduled caesarian section under a IRB-approved protocol, was used.

*Enzymatic degradation of amniotic membrane.* Crosslinked and control AM was digested with Type 1 collagenase at 37°C for 5 min to 20 hr. The increase in peptide free amino groups that are formed by cleavage of peptide linkages in AM proteins was measured using a fluorescence assay. The percent degradation of collagen in crosslinked AM was compared to tht in untreated controls.

*Photochemical crosslinking.* We evaluated two dye photosensitizers (riboflavin and Rose Bengal) and UVC (germicidal) radiation. Two types of results were obtained: 1) Maximum percent inhibition of degradation relative to untreated control, and 2) Kinetics for the degradation of the crosslinked AM compared to untreated AM.

*Riboflavin (RF) photosensitization* used a water soluble form of riboflavin. RF with UVA irradiation has recently been used to crosslink corneal collagen as a treatment for keratoconus (1) indicating that it is a safe photosensitizer. The maximum percent inhibition using RF and blue light was 35.5 ± 12 (Fig. 1A). The kinetics of enzymatic degradation of crosslinked AM were substantially slower than for untreated AM (Fig. 1B). These graphs are shown as representative of the results obtained with all the crosslinking methods tested. Graphs for the other methods are shown in Year 1 report, Figs. 2-7.

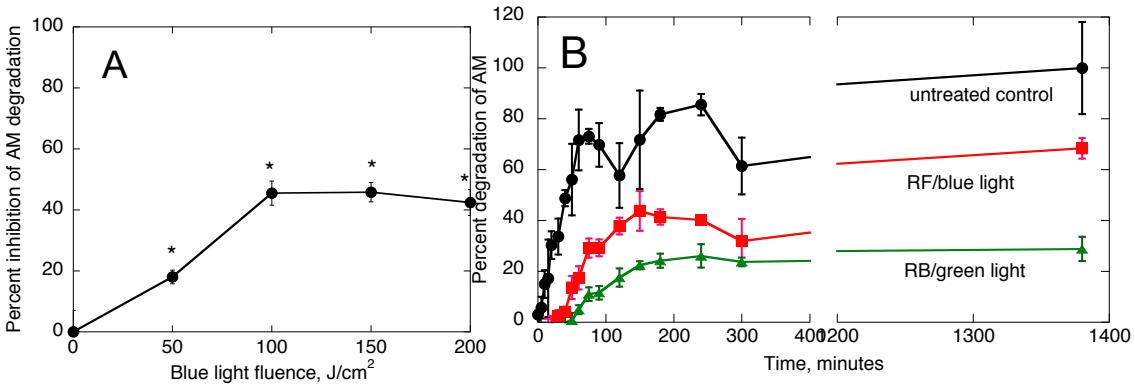


**Figure 1.** Representative results showing decrease in enzymatic degradation of amnion after crosslining AM. (A) Riboflavin/blue light treatment. Percent inhibition of collagenase digestion after RF photosensitization of AM with varying fluences of blue light. * $p < 0.05$ compared to untreated control AM. (B) Kinetics of collagenase-induced degradation of AM. Black circles = untreated AM. Red squares = AM treated with RF and 200 J/cm$^2$ blue light. Green triangles = AM treated with RB and 10 J/cm$^2$ green light.

*Rose Bengal (RB) photosensitization* was tested because RB is FDA-allowed as a diagnostic for corneal abrasions suggesting that it will be acceptable for AM photo-crosslinking. The maximum percent inhibition for RB-photosensitized collagen crosslinking was 55 ± 13 (Table 1) and the kinetics shown in Fig. 1B indicate that RB protected AM against enzymatic degradation even more effectively than RF photosensitization.

*Ultraviolet radiation (germicidal)* has been used to crosslink tissue proteins, but has been associated with breakdown of protein chains in some cases. The maximum percent degradation was 48% level and the initial rate of AM degradation was ~2-fold lower for the membranes treated with UVC.

*Chemical crosslinking.* We evaluated three chemical agents that crosslink proteins by different mechanisms: glutaraldehyde, carbodiimide and genipion.

*Glutaraldehyde* is a well known and effective crosslinking agent for structural proteins (2). At a concentration of 25 μM, it produced nearly 100% inhibition of degradation. However, it was not investigated further because of reports of toxic side products.

*Carbodiimide* (EDC) was tested because it has been shown to enhance the physical properties of AM for use as a scaffold in tissue engineering (3). This approach to chemical crosslinking of proteins has the advantage that only non-toxic, water-soluble urea derivatives are side products. Total inhibition of degradation was achieved using 20 mM carbodiimide (Table 1).

*Genipin* is an excellent natural protein crosslinker with very low toxicity. Treating AM with 0.1% genipin for 20 h produced a maximum percent inhibition of 86 ± 8.

**Table 1.** Summary of results from testing the ability of protein crosslinking agents to protect amniotic membrane against enzymatic degradation.

| Crosslinking treatment | Treatment conditions | Number of trials | Maximum percent inhibition of degradation [a] |
|---|---|---|---|
| Control, no treatment | ---- | 25 | --- |
| RF-5P, 1% | 50-200 J/cm$^2$ | 10 | 35 ± 12 at 100 J/cm$^2$ |
| RB, 0.1% | 2.5-20 J/cm$^2$ | 10 | 55 ± 13 at 10 J/cm$^2$ |
| UVC | 15-120 J/cm$^2$ | 4 | 43 ± 6 at 120 J/cm$^2$ |
| Glutaraldehyde | 10-50 μM, 30 min | 4 | 100 ± 3 at 50 μM |
| Carbodiimide (EDC) | 1-20 mM, 24 hr | 3 | 100 ± 5 at 20 mM |
| Genipin | 0.1%, 2-20 hr | 5 | 86 ± 8 at 0.1% |

[a] Samples were incubated with 1% collagenase at 37°C for 20 hr.

The three chemical crosslinking methods (glutaraldehyde, carbodiimide and genipin) produced materials that were the most resistant to enzymatic degradation. We also continued to consider photosensitization with RB because it blocked more than half of the enzymatic degradation. Glutaraldehyde was not evaluated further because of reports of toxic byproducts.

We observed that highly crosslinked AM appeared to be stiffer than untreated AM and thus did not conform to the shape of the cornea. Since this would be a problem for covering and protecting ectropion cornea, we quantitatively evaluated the stiffness of the AM crosslinked by carbodiimide, genipion and RB photosensitization. The stiffness was compared to the percent enzymatic degradation of the same membrane. The elastic modulus of untreated and crosslinkd AM was calculated from stress-strain curves obtained from uniaxial tensiometry measurements.

The stiffness of the AM samples was then calculated. Figure 2 shows an example of the results obtained (other results are shown as Figures 1-3, Year 3 report).

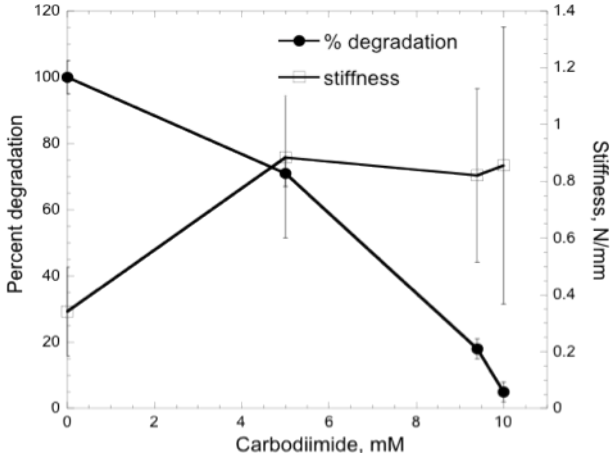


**Figure 2.** Effect of crosslinking monolayer amnion with carbodiimide on degradation by bacterial collagenase and on membrane stiffness. Amnion samples were incubated for 1 h with varying concentrations of carbodiimide, then degraded with bacterial collagenase or used for uniaxial tensiometry measurement of the elastic modulus, which was used to calculate stiffness.
* indicates $p < 0.01$ compared to control (no carbodiimide).

This graph shows that percent enzymatic degradation and stiffness show different patterns of response to treatment with increasing carbobiimide concentration. The stiffness reaches a plateau starting at the lowest carbodiimide concentration (5 mM). Thus, the higher concentrations of carbodiimide, which produce nearly complete protection of the amnion from degradation, do not further increase the amnion stiffness. This is good news since it means that we can vary the degree of protection against enzymatic degradation without causing the monolayer membrane to be too stiff to conform to the shape of the cornea. A similar result was found for crosslinking amnion with genipin. RB photosensitization produced less stiffness, although less protection against enzymatic degradation.

*In summary*, by the criteria of ability to inhibit degradation of AM proteins and to produce a pliable membrane, three crosslinking methods (RB photosensitization, carbodiimide and genipin) were considered to be suitable for forming a membrane suitable for placement over the cornea of burn patients.

1.b. Determine whether a multilayer composite of amnion retards the rate of proteolytic degradation in vitro. An alternative approach evaluated for stabilizing AM against enzymatic degradation is increasing the thickness of the membrane by sealing together a stack of AM layers. The hypothesis is that the protein in the center of this thicker membrane would be less accessible to proteolytic enzymes; consequently, the time required to degrade the amnion would increase. Multilayer AM constructs were produced by sealing the layers together using two protein crosslinking methods, carbodiimde (EDC) and Rose Bengal photosensitization.

Composites of 2 and 3 AM layers were constructed by keeping the AM layer surfaces in tight contact while treating with a crosslinking method. The percent degradation in the layered composites compared to the control (not treated with 5 or 10 mM carbodiimide) is shown in Fig.3. The trilayer membrane crosslinked with 5 mM carbodiimide was more resistant to proteolytic degradation than the similarly-treated monolayer, consistent with the hypothesis that the thicker membrane protects AM proteins from proteolytic degradation. This result is supported further by the results obtained using 10 mM carbodiimide; both the bi- and trilayer membranes showed less proteolytic degradation than the monolayer membrane. Similar experiments using RB photosensitization to form bi- and tri-layer composites also indicated that

the trilayer composites were almost totally resistant to enzymatic degradation (Fig. 3, Year 2 report.)

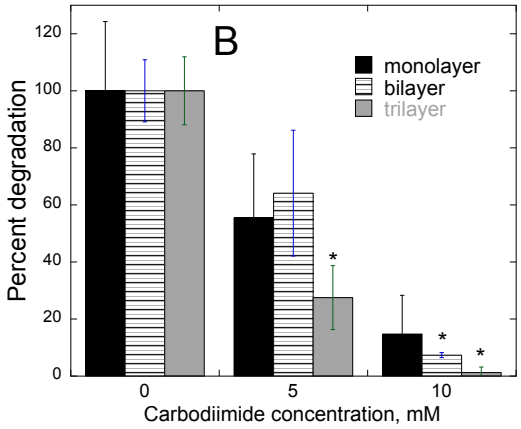


**Figure 3**. Comparison of the susceptibility of mono-, bi- and trilayer amnion composites to proteolytic degradation after crosslinking with 5 and 10 mM carbodiimide. * indicates $p < 0.05$ compared to monolayer amnion treated with the same carbodiimide concentration

Although the trilayer AM composite met the criteria of blocking enzymatic degradation, they were too stiff and could not be applied to conform to the cornea surface. This was confirmed by tensiometry measurements; the stiffness was 1.44 ± 0.51 N/mm, substantially higher than the stiffness of the monolayer amnion (Table 1, Year 3 report).

1.c. Assess biochemical and structural alterations in cornea of rabbit model for ectropion. Create ectropion by blepharoplasty in New Zealand white rabbits. Assess epithelial defects and corneal ulcers. Measure inflammatory cytokines and proteolytic enzymes.

The partnering PI, COL Anthony J. Johnson MD, has created this model. The results are described in his Annual Reports.

1.d. Determine whether the relative degradation rates of modified amnion in vivo correlate with those measured in vitro. Amnion with differing degrees of protein crosslinking will be secured to rabbit cornea. The loss of amnion will be followed by slit lamp examination and histology.

These studies are ongoing in Dr. Johnson's laboratory at USAISR and will be completed during an extension of his grant period. Amnion, untreated and crosslinked, has been transferred to his facility for these studies. We will supply additional samples as needed.

1.e. Identify the protein crosslinking method that has causes the least reduction in anti-inflammatory and healing factors in amnion. Amniotic membrane contains several growth factors and cytokines believed to contribute to the pro-healing and anti-inflammatory properties of aminon (4, 5). We chose to test the effect of protein crosslinking methods two beneficial proteins that represent different localization in the AM stroma. One, TGF-β1, is in the group that are stored in the stroma tightly bound to extracellular matrix proteins; the crosslinking chemistry is likely to form links between the matrix proteins and TGF-β1. The other, EGF, is representative of small proteins that is not stored in close association with extracellular matrix proteins in tissue. Proteins were extracted from untreated and crosslinked AM, then TGF-β1 and EGF were assayed by ELISA. Acid activation was used to release of TGF-β1 prior to ELISA.

*The effect of crosslinking on TGF-β content* of AM was measured after treatment with carbodiimide (EDC) and RB photosensitization. Representative results are shown in Fig.4 (see

Figs. 4, 5, Year 2 report). The percent degradation of AM and the TGF-β content were measured for samples treated with carbodiimide under varying conditions. These results

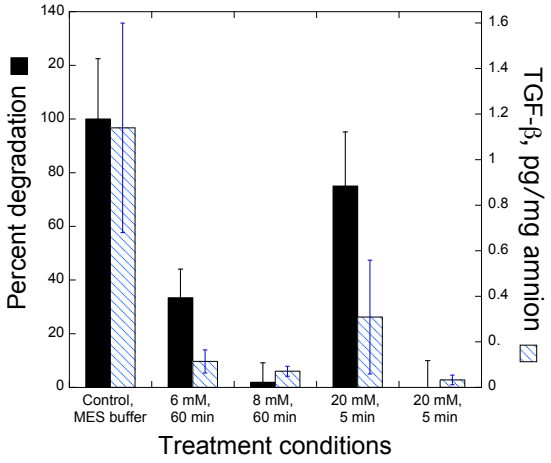


**Figure 4.** Effect of crosslinking amnion proteins with carbodiimide on the level of TGF-β. The percent proteolytic degradation of treated and control amnion is shown as black bars. The level of TGF-β was measured on the same samples by ELISA (blue stripped bars).

show that treatment with 8 mM carbodiimide for 60 min and with 20 mM for 5 min completely blocked enzymatic degradation, consistent with our previous results. However, all the treatments markedly or completely blocked TGF-β1 availability. This result indicates that the carbodiimide-treated amnion, although it is resistant to enzymatic degradation, does not retain the native level of available TGF-β1. Similar experiments were carried out using RB photosensitization to crosslink AM proteins. At the lower fluence, which decreased degradation by ~50%, almost 30% of the TGF-β1 was still available.

*Next, the effect of crosslinking on available EGF content* of AM was measured after treatment with carbodiimide (EDC), RB photosensitization and genipin. Representative results obtained after treatment with genipin are shown in Figure 5. (See Figs. 2-5, Year 3 Report for additional results). Crosslinking with carbodiimide decreased both the percent enzymatic degradation and the EGF content of the amnion; the EGF content decreased more rapidly than the percent degradation. Photo-crosslinking using RB photosensitization decreased the available EGF level although about 20% remained even in the most highly crosslinked samples. The reduction in EGF was partially attributable to RB alone (no light). Amnion was treated for varying times with 0.1% genipin in PBS. As shown Figure 5, the decrease in EGF paralleled the decrease in percent degradation. Thus, genipin crosslinking of AM produced a material with excellent resistance to enzymatic degradation but without available EGF.

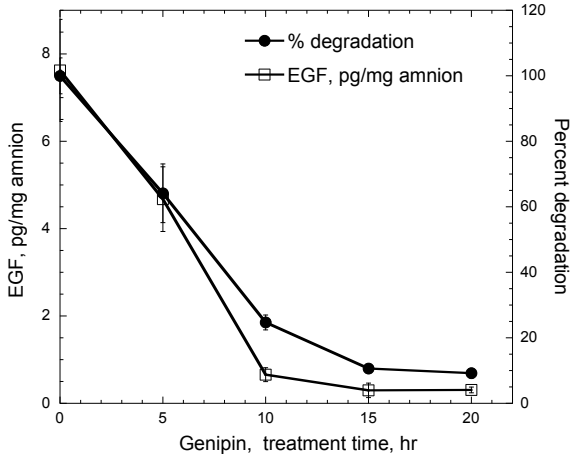


**Figure 5** Effect of crosslinking monolayer amnion with genipin on EGF and enzymatic degradation. Amnion was incubated for varying times with 0.1% genipin.
* indicates $p < 0.01$ compared to MES buffer control

Since tri-layer membranes might provide a method of protecting the EGF in the inner AM, similar measurements were made after using carbodiimide (3 to 20 mM) for producing tri-layer AM. Although the amount of EGF extracted decreased with increasing carbodiimide concentration, at 5 mM and higher tri-layer membranes had higher EGF than monolayers suggesting that the EGF in the middle layer was protected. Although this was an interesting and useful observation, the stiffness of the tri-layer (as described above), does not allow it to be considered further as a protective membrane for ectropion eyes.

*In summary,* both carbodiimide and genipin crosslinking were rated highly on the criteria of blocking enzymatic degradation of AM and ability to conform to the contour of the cornea surface, both methods decreased almost totally the availability of TGF-β1 and EGF in the AM. Some residual TGF-β1 and EGF was available after RB photosensitized crosslinking and the AM remains pliable; the disadvantage of this method is that only ~50% inhibition of degradation is produced. Tri-layer amnion crosslinked by carbodiimide treatment showed better retention of EGF than similarly crosslinked monolayer membranes. However, the high stiffness of these enzyme-resistant crosslinked tri-layers precludes their use for cornea protection.

1.f. Evaluate the healing properties of crosslinked amnion and layered amnion using the rabbit ectropion model.

The rabbit ectropion model has been established by Dr. Johnson at USAISR as described in his annual report. We have prepared and sent samples of monolayer amnion crosslinked with carbodiimide and untreated controls to Dr. Johnson to be tested on these rabbits. We will continue to supply crosslinked amniotic membranes to Dr. Johnson. The tri-layer AM constructs crosslinked with carbodiimide were too stiff to conform to the contour of the cornea. Consequently they will not be evaluated in the rabbit ectropion model created by Dr. Johnson.

**Specific aim 2. To determine whether a photoactivated method for bonding amnion to cornea provides a rational alternative to suturing.** (Results in Year 2 report, Figs. 6 and 7).

The crosslinked, degradation-resistant AM that is being developed in this project could be placed on the inflamed eye in a Prokera®–like device or be sutured to the cornea as is done in AM transplantation. However we have demonstrated (not part of this project) that untreated AM can be tightly attached to normal cornea using RB and green light; the AM crosslinks with proteins on the cornea surface. We have not tested whether pre-crosslinked AM, produced by the methods of the current project, can be photobonded to cornea using the photobonding method described in our previous study (6).

Carbodiimide was used to crosslink 13-mm diameter discs of AM, which were then were stained with RB and placed on the cornea (containing an penetrating incision) of ex vivo rabbit eyes. Green light was used to irradiate the AM. The bonding strength was measured by infusing a saline solution containing a blue dye into the anterior chamber and measuring the pressure we have done previously (7). The maximum pressure attained before leakage from under the amnion is observed is termed the leak intraocular pressure ($IOP_L$) and is reports on the bonding strength.

Membranes that were pre-crosslinked with 10 mM carbodiimide showed the same $IOP_L$ as the non-pre-crosslinked control membranes when exposed to 50 and 100 J/cm$^2$ indicating that pre-crosslinking did not hinder bonding of AM to the cornea surface. Thus,10 mM carbodiimide-treated amnion, which is highly resistant to enzymatic degradation, can be photobonded to cornea. This result supports the hypothesis that a crosslinked, degradation-resistant AM can be

securely sealed to cornea by photobonding. Similar experiments were carried out with RB photosensitized crosslinking prior to bonding the AM to the cornea surface. The results showed that pre-crosslinking treatment with RB/green light did not alter the ability of photobonding to seal AM over the corneal wound.

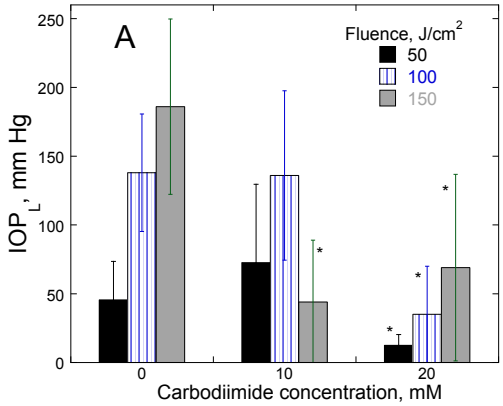


Figure 6. Influence of pre-crosslinking amniotic membrane proteins on the photobonding of amnion over a full thickness incision in the corneal surface. The strength of bonding between amnion and cornea was measured as the anterior chamber pressure that causes disruption of the bond ($IOP_L$). (**A**) Amnion pre-crosslinked with carbodiimide, then photobonded to cornea. Asterisk indicates $p<0.05$ compared to control, non-crosslinked amnion at the same photobonding fluence

*In summary,* these results indicate that pre-crosslinked AM that is resistant to enzymatic degradation can be tightly sealed to the cornea surface. This technique may provide a viable alternative to sutures for securing degradation-resistant amnion to cornea for treatment of cornea of ectropion eyes.

**Specific aim 3. To combine amnion with a water-retaining layer of material to provide hydration to the corneal surface.** Use photochemical crosslinking to seal hydrophobic material within an amnion covering.

Currently corneal hydration for patients with cicatricial ectropion is maintained by frequent administration of artificial tears. Amnion, by itself, does not supply moisture to the cornea. Consequently, we encapsulated a hydrogel contact lens within an amnion capsule to provide a source of moisture for the cornea. Bonding this construct to the cornea using a light-activated process would keep the contact lens construct in place and prevent bacterial infection under the contact lens.

A hydrophilic hydrogel contact lens was sealed between two discs of AM. One of the discs was stained with a 2 mm band of RB on the edge. When the two discs were in contact at the edges, they were exposed to green light to crosslink the AM layers. (The light activates the RB dye around the perimeter of the amnion thereby photocrosslinking proteins between the two amnion surfaces (Step 4).

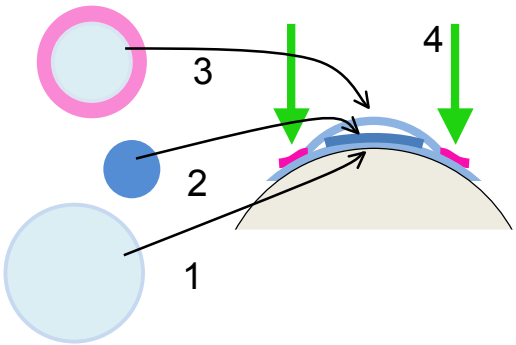


Figure 8. Encapsulating a hydrophilic contact lens with amniotic membrane. (1) Amnion disc (18 mm) placed on hard sphere. (2) Contact lens placed on amnion disc. (3) Amnion (15 mm) with RB-stained perimeter placed over lens and lower amnion. (4) RB-stained amnion is irradiated with 532 nm (green light) to seal the two amnion layers together.

To test the integrity of the seal around the contact lens, a 25-gauge needle was inserted into the chamber containing the lens, and blue dye solution was slowly pumped in. The pressure within the chamber was monitored and reached values between 2 and 110 mm Hg (n = 18) before leakage occurred.

Preliminary tests were carried out to determine whether the amnion-encapsulated contact lens construct would bond to a cornea ex vivo. The bottom amnion-covered surface of the construct was stained with RB (0.1% for 5 min). This was then placed on the de-epithilialized cornea surface of an ex vivo rabbit eye containing a V-shaped incision and irradiated. Measurements of the leak intraocular pressure on 5 eyes gave a value of 87 ± 41 mm Hg. This pilot study result indicates that the concept of bonding a hydrating contact lens to the cornea is potentially feasible.

Similar pilot studies were carried out using carbodiimide as the crosslinking agent but did not produce an encapsulated lens. Our initial studies, reported in year 3 report, showed some success but studies carried out in the no-cost-extension period indicated that carbodiimide crosslinking did not consistently seal the AM layers around the lens

*In summary,* construction of an amnion-encapsulated hydrophilic lens is feasible using RB photo-crosslinking to seal the capsule. Preliminary studies suggest that these constructs can be bonded to the cornea. These constructs may be an alternative method for providing hydration to the cornea.

**KEY RESEARCH ACCOMPLISHMENTS**

- Identified the crosslinking treatment conditions for six methods that provide the maximum protection against enzymatic degradation. The six methods were selected because they form crosslinks by different molecular mechanisms and consequently form different crosslinks that may differ in their ability to protect amniotic membrane against enzymatic degradation.

- The relative levels of maximum protection against enzymatic degradation of amniotic membrane were found to be: Glutaraldehyde = carbodiimide = genipin > Rose Bengal photosensitization > UVC radiation > riboflavin photosensitization.

- Established that 2 and 3 layer crosslinked amniotic membrane composites are more resistant to enzymatic degradation than monolayer amnion.

- Determined that crosslinking of amnion proteins using a carbodiimide (EDC) and genipin cause almost total loss of available TGF-$\beta$1 and EGF, two beneficial small proteins in amnion. Rose Bengal photosensitization produced membranes that retained ~20% of these small proteins but inhibited enzymatic degradation by only ~50%.

- Identified stiffness of crosslinked amnion membranes as a critical physical property for developing a protective membrane since the membrane must conform to the shape of the cornea. Amnion crosslinked with carbodiimide, genipin or Rose Bengal photosensitization were both sufficiently low in stiffness (high pliability) and sufficiently resistant to enzymatic degradation for be tested in vivo.

- Determined that the tri-layer crosslinked amnion partially retained EGF in the membrane but was unsuitable as a protective membrane because of its high stiffness.

- Demonstrated that crosslinked, degradation-resistant amnion can be photobonded to cornea thereby providing an alternative for suturing or a retaining ring to secure these protective membranes to the ocular surface.

- A protease-resistant amnion-covered hydrogel contact lens for providing hydration to the cornea was constructed using Rose Bengal photosensitization in pilot studies.

## REPORTABLE OUTCOMES

I.E. Kochevar, E. Verter, T. Gisel, R.W. Redmond, A.J. Johnson, Corneal Protection for Burn Patients, Medical Health Services Research Symposium, Aug 12-16, 2012, Fort Lauderdale, FL, Poster 12-015. A video presentation of this poster was featured as a video highlight on the CDMRP home page (http://cdmrp.army.mil).

A.J. Johnson, P. Buttke, I. Kochevar, H-C.Wang, S. Cora, S. DeMartelaere, Surgical Model for Evaporative Loss Dry Eye Model in the New Zealand White Rabbit, Association for Research in Vision and Ophthalmology, June 10-16, 2013, Seattle WA. Poster 6046—A0109.

## CONCLUSIONS

The major goal of this research was to develop a modified amniotic membrane (AM) that can be placed on the cornea of burn patients with cicatricial ectropion to prevent desiccation and development of corneal defects, inflammation, infection and opacification. Unmodified AM is rapidly degraded by proteases in the tears of these inflamed eyes. Our approach was to crosslink proteins, largely collagen, in the AM, which would decrease the rate enzymatic degradation. We used four criteria to evaluate the suitability of crosslinking methods: 1) maximum percent inhibition of enzymatic degradation after crosslinking, 2) flexibility of the crosslinked AM that allows it to conform to the shape of the cornea (measured as stiffness), 3) retention of beneficial factors (cytokines/growth factors) found in native amnion, and 4) good safety profile based on literature reports.

These four criteria were used to evaluate six crosslinking methods and a method to form multilayer membranes. Two crosslinking methods (UVC irradiation and riboflavin photosensitization) were eliminated because of low protection against enzymatic degradation. Gutaraldehyde was eliminated because of reports of toxic side products produced during crosslinking tissue. The multilayer method was eliminated because the material produced was too stiff.

Three methods were identified as having potential to be tested in vivo by our collaborating PI, Dr. Anthony Johnson. None is ideal, i.e., having all the properties desired in an ideal material, but all have sufficiently good profiles to be tested in vivo. Rose Bengal photosensitization was not optimal for blocking enzymatic degradation but remained flexible and retained availability of some of the two beneficial factors assayed (TGF-$\beta$1 and EGF). Crosslinking with a carbodiimide (EDC) and genipin totally blocked enzymatic degradation, remained reasonably flexible but caused total loss of available TGF-$\beta$1 and EGF. Carbodiimide crosslinking was

chosen over genipin crosslinking for in vivo evaluation because the carbodiimide process is shorter and the reagents are less expensive. Thus, AM crosslinked with RB photosensitization and carbodiimide (EDC) will be evaluated. Dr. Johnson is currently conducting these studies using the ectropion model he developed. The results of those studies will determine whether this approach to developing a protective membrane for cornea of ectropion eyes has the potential to be extended to patients.

Our studies also demonstrated that crosslinked, degradation-resistant AM can be securely sealed to the corneal surface using a sutureless, light-activated technique. This approach may be an improvement over the current requirement of a ring on the sclera or sutures to hold the AM covering in place.

We also demonstrated in a pilot study that an amnion-covered hydrophilic lens can be constructed using photochemical crosslinking of two layers of AM and sealed to the cornea using photobonding. Although considerable development is needed for this device, it would provide hydration to the cornea and thus inhibit damage to the ocular surface.

**REFERENCES**


1. Wollensak, G., E. Spoerl and T. Seiler (2003) Riboflavin/ultraviolet-a-induced collagen crosslinking for the treatment of keratoconus. *Am J Ophthalmol,* 135, 620-7.
2. Fujisato, T., K. Tomihata, Y. Tabata, Y. Iwamoto, K. Burczak and Y. Ikada (1999) Cross-linking of amniotic membranes. *J Biomater Sci Polym Ed,* 10, 1171-81.
3. Ma, D. H., J. Y. Lai, H. Y. Cheng, C. C. Tsai and L. K. Yeh (2010) Carbodiimide cross-linked amniotic membranes for cultivation of limbal epithelial cells. *Biomaterials,* 31, 6647-58.
4. Dua, H. S., J. A. Gomes, A. J. King and V. S. Maharajan (2004) The amniotic membrane in ophthalmology. *Surv Ophthalmol,* 49, 51-77.
5. Koizumi, N. J., T. J. Inatomi, C. J. Sotozono, N. J. Fullwood, A. J. Quantock and S. Kinoshita (2000) Growth factor mRNA and protein in preserved human amniotic membrane. *Curr Eye Res,* 20, 173-7.
6. Verter, E. E., T. E. Gisel, P. Yang, A. J. Johnson, R. W. Redmond and I. E. Kochevar (2011) Light-initiated bonding of amniotic membrane to cornea. *Invest Ophthalmol Vis Sci,* 52, 9470-7.
7. Proano, C. E., D. T. Azar, M. C. Mocan, R. W. Redmond and I. E. Kochevar (2004) Photochemical keratodesmos as an adjunct to sutures for bonding penetrating keratoplasty corneal incisions. *J Cataract Refract Surg,* 30, 2420-4.


**Personnel supported by this grant**

All personnel are employed by the Massachusetts General Hospital, Boston MA.

Irene E. Kochevar, PhD, Principal Investigator
Robert W. Redmond, PhD, Investigator
E. Eri Verter, MS, Senior Technologist
Thomas Gisel, MS, Senior Technologist
Francis Doyle, MS, Technologist

## CORNEAL PROTECTION FOR BURN PATIENTS

**Irene E. Kochevar, Ph.D.**

**PURPOSE/AIMS:** The goal of this research was to develop a material to protect the cornea of patients recovering from severe facial burns and thereby to help preserve their vision.

**DESIGN:** Human amniotic membrane (HAM) can provide a healing environment to damaged cornea. However, it is very rapidly degraded by proteolytic enzymes in tears of the inflamed eyes of patients with ectropion who cannot blink because of severe facial burn scars. We hypothesized that crosslinking proteins in HAM would produce a degradation resisant, effective material for cornea protection. Inhibition of enzymatic degradation, potential loss of beneficial cytokine and growth factors, and tensile strength of the crosslinked HAM were measured in vitro to begin to test this hypothesis.

**METHODS:** Proteins in HAM were crosslinked using five methods that differ in their chemical mechanisms and type of crosslink formed. Bacterial collagenase was used for enzymatic degradation. The effects of protein crosslinking on the rate and extent of enzymatic degradation were quantitated using a fluorescence-based assay measuring products of proteolysis (free peptides). Transforming growth factor-β (TGF-β) and epidermal growth factor (EGF) in HAM were measured by ELISA. OCT microscopy was used to measure HAM thickness. Young's modulus, calculated from uniaxial tensiometry measurements, was used to evaluate stiffness.

**DATA ANALYSIS:** Student's t-test for unpaired samples was used to compare groups with significance set at $p < 0.05$.

**FINDINGS:** Proteolytic degradation rates and total degradation after 24 hr collagenase treatment decreased after all crosslinking methods. The maximum percent degradation decreased in the order: glutaraldehyde (100%) = carbodiimide (100%), Rose Bengal photosensitization (55%), germicidal UVC (43%), riboflavin photosensitization (35%). Levels of TGF-β and EGF were significantly reduced by crosslinking. Young's modulus increased by up to 2-fold. Carbodiimide crosslinking gave the best combination of resistance to degradation, non-toxicity and acceptable stiffness for covering cornea.

**CONCLUSIONS/RECOMMENDATIONS:** Proteolysis-resistant crosslinked HAM may be a suitable material for covering and protecting inflamed cornea of burn patients. Evaluation of this material in an animal model for ectropion is warranted.

**IMPLICATIONS:** A protective membrane for cornea of burn patients with severe facial scarring could prevent corneal erosion, scarring and potentially loss of vision.

**FROM/TO TIME PERIOD OF STUDY:** 01-07-2009 to 01-04-2012
**FUNDING:** PRMRP Contract No. W81XWH-09-2-0070

**Assoication for Research in Vision and Ophthalmology,**
**2013 Annual Meeting**
**Abstract 6046—A0109**

# Surgical Model for Evaporative Loss Dry Eye Model in the New Zealand White Rabbit

**Anthony Johnson[1,3], Patricia Buttke[3], Irene Kochevar[2], Heuy-Ching Wang[1], Steven Cora[1] and Sheri DeMartelaere[3]**
[1] Ocular Trauma Research, USAISR, Fort Sam Houston, TX
[2] Wellman Center for Photomedicine, Massachusetts General Hospital, Boston, MA
[3] SAUSHEC Ophthalmology, San Antonio Military Medical Center, Fort Sam Houston, TX

**Purpose:**Patients with severe facial burns often suffer indirect damage to their eyes. Burn wound contracture of the periocular skin causes cicatricial ectropion resulting in ocular exposure. Skin grafts are often required, but may be insufficient if deeper structures, such as the periocular muscles, are injured and the protective blink reflex is lost. With loss of the blink reflex the patient quickly develops exposure keratitis. No current treatment adequately addresses the severe keratitis that these patients develop. We established this evaporative loss dry eye model that simulates exposure keratopathy resulting from cicatricial ectropion to assist in the development of novel therapies for this condition.
**Methods:**Nine white rabbits were included in this study. The right eye of each rabbit was subjected to a 1.5 cm upper and lower lid blepharoplasty, in addition to excision of the nictitating membrane. The left eyes were untreated to serve as controls. Clinical examination included fluorescein staining and serial photography on days 3, 5, 7, 14, 21 and 28. Rabbits were sacrificed on day 28 and the cornea and conjunctiva were evaluated by histopathology.
**Results:**Compared with untreated controls, surgically treated rabbits showed significant changes in fluorescein scores on days 5, 7, 14, and 28. Clinical examination revealed ocular surface defects ranging from the development of punctate epithelial erosions to corneal abrasion, beginning on day 7, with corneal ulceration developing in the most severe cases by week 3. Histopathological results revealed epithelium infiltrated by heterophilic inflammation with the underlying corneal stroma demonstrating heretophilic, lymphoplasmacytic inflammation, fibrosis and neovascularization.
**Conclusions:**The findings of this study demonstrate that upper and lower lid blepharoplasty combined with excision of the nictitating membrane is an excellent surgical model of evaporative dry eye. Damage to the cornea and conjunctiva manifesting as punctate epithelial erosions and corneal ulceration is consistent with clinical observations of burn patients with dry eye resulting from cicitricial ectropion. Future studies utilizing this model are planned to test multiple strategies to protect the ocular surface from evaporation due to ocular exposure.